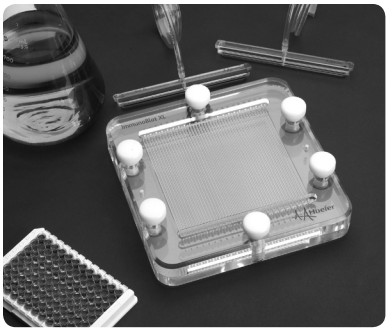
user manual

ImmunoBlot and ImmunoBlot XL

Operating instructions



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Introduction

Transfer of proteins or nucleic acids, fractionated by gel electrophoresis, to an immobilizing membrane surface increases the sensitivity of a wide range of detection methods, reduces time of analysis and makes sequential probing possible. In particular it has made possible use of immunological procedures that are not practical to carry out in a gel. To assay material on a sheet of membrane with different probes simultaneously commonly requires first cutting the membrane into a number of parallel strips. This procedure however destroys the correspondence between different lanes or positions on a gel.

The ImmunoBlot® and ImmunoBlot XL preserve this correspondence while minimizing the volume of detection reagents needed for individual lanes. Two clear plastic plates, one smooth and one with channels, clamp a 15 × 15 cm transfer membrane to define and separate the original gel lanes into individual channels. These separate channels allow for the use of different detection reagents (primary antibodies, conjugated secondary antibodies, antigens and/or reaction substrates) in each channel.

There are 2 principal applications for the ImmunoBlot and ImmunoBlot XL:

Multiple antisera or probes may be tested against a single sample of protein or nucleic acids. The single sample is loaded across the entire top of a slab gel, then run and blotted to a membrane. When clamped to the ImmunoBlot, the membrane can be tested against multiple antisera or probes.

A single antiserum or probe can be tested against multiple antigens or nucleic acids.

The two models differ in the number and volume of the reaction channels. The ImmunoBlot has 25 channels that hold a maximum volume of 250 µl each. The ImmunoBlot XL has 45 channels that hold a maximum volume of 140 µl each.

Unpacking and inventory

Unwrap all packages carefully and compare contents with the packing list, making sure all items arrived. If any part is missing, contact your local sales office. Inspect all components for damage that may have occurred while the unit was in transit. If any part appears damaged, contact the carrier immediately. Be sure to keep all packing material for damage claims or to use should it become necessary to return the unit.

Instructions

The following detail the steps for probing a membrane using the ImmunoBlot.

Select and block membrane

❶

Select a membrane of choice (nitrocellulose, Hybond ECL™; PVDF, Hybond P; low fluorescent PVDF, Hybond LFP) for the assay and cut to:

Length: 15 cm

Width: Cut to accommodate channels to be used
Maximum width for all channels: 15 cm

❷

Block membranes with excess non-specific protein.[1]

❸

Note: If blocked membranes have been stored in a protein-free solution, re-block them for 1 to 2 minutes in a tray of blocking solution.

Load membrane

❶

Remove the top acrylic plate of the ImmunoBlot and turn it over.

❷

With the antigen-bearing face of the membrane facing the ImmunoBlot channels, position the membrane so it covers all the channels.[2]

❸

Place a new, dry sealing pad (supplied with unit) over the membrane.

❹

Set the bottom plate of the unit on the inverted top plate so the alignment pins fall in place.

❺

Ensure there is no gap between the top and bottom plates.

[1]See Appendix A, Note 1, Blocking the membrane.

[2]See Appendix A, Note 2, Aligning the membrane.

Aspirate and introduce antibody solutions

Recommendation: Use a disposable pipette tip connected to a water vacuum aspirator for this purpose.

Important! Take care not to touch the pipette tip anywhere on the unit except in the desired sample hole.

❶

Aspirate excess liquid from the channels through the numbered holes.

Note: To avoid drying of the membrane, channels should be loaded within five (5) minutes of aspirating.

❷

Pipette antibody solution through the numbered holes.

Press the pipette tip firmly into each hole and inject the liquid rapidly in a single smooth action until the channel is filled. Take care not to introduce bubbles.[3]

❸

Add buffer to all unused channels that cover the membrane.

model	number of channels	approximate channel volume[4]
ImmunoBlot	25	250 µl
ImmunoBlot XL	45	140 µl

Incubate

❶

Place the unit on a rocking platform with the channels aligned in the direction of rocking.

Note: For best results, use a slow rocking speed (5 to 6 tilt cycles per minute). A gyratory shaking platform is not effective for this purpose.

❷

Incubate the unit on the rocking platform for 30 to 60 minutes at room temperature.

[3]See Appendix A, Note 3, Eliminating bubbles.

[4]See Appendix A, Note 4, Optimal volumes.

Remove primary antibody solutions and wash the blot

Use the washing manifold to remove the antibody solution and wash the blot.

❶

Position the 2 identical parts of the washing manifold in the slots on either side of the top plate and press firmly until the O-rings are seated in the slots.[5]

❷

To aspirate all samples simultaneously, first connect pieces of the tubing supplied with the unit to both parts of the manifold using a luer fitting. Now, connect the open end of the tubing from the manifold piece to a trap connected to a vacuum source.

Then place the open end of the tubing from the second manifold piece into a beaker containing wash buffer.

When you start the vacuum antibody solutions in the channels are removed and the wash buffer is drawn in from the other side. Please refer to your Western detection protocol for wash instructions. For Western detection we recommend at least 2 quick washes in buffer followed by two longer washes in buffer (5 min while rocking is sufficient) before introducing the secondary antibodies.

[5]See Appendix A, Note 5, Fitting the O-rings into the slots.

Introduce secondary antibody and incubate

The secondary antibody incubation may be performed either in the unit or in a tray.[6]

To perform the incubation in the ImmunoBlot unit:

❶

Inject the secondary antibody solution into the channels carefully with a single or multi-channel pipette.

❷

Incubate the unit on a rocking platform with the channels aligned in the direction of rocking for 30 to 60 minutes at room temperature.

❸

Aspirate the secondary antibody solution using a vacuum source or pipette as described before (page 5, step 2) and wash the blot with wash buffer for a few seconds. Washing the blot prevents cross contamination of channels by different secondary antibodies across channels when removing the blot from the unit.

[6]See Appendix A, Note 6, Secondary antibody incubation.

Remove and develop the blot

❶

Unscrew the ImmunoBlot and separate the two halves.

❷

Discard the used sealing pad and wash the membrane in a tray with 3 to 5 changes of buffer for a total of 10 to 15 minutes.

❸

Develop the blot by following instructions from your Western detection kit (See also Appendix B).

Clean the ImmunoBlot

Clean the unit thoroughly after each use by performing the following:

❶

Rinse the ImmunoBlot unit and washing manifold under distilled water.

❷

Use a non-abrasive, alkaline laboratory cleaning agent that leaves no residue after rinsing, such as a detergent intended for cleaning tissue culture glassware. Concentrated cleaning agents should be diluted to normal strength.

Recommendation: A 1% solution of 7X Cleaning Solution (Flow Laboratories, 50 ml concentrate in 5 liters of warm water).

❸

Immerse the ImmunoBlot unit and washing manifold in the cleaning solution and brush gently with a soft brush taking care not to scratch the interior surfaces.

❹

Rinse the ImmunoBlot unit and washing manifold thoroughly with tap water, followed by distilled water.

To easily rinse the small numbered holes, assemble the unit and manifold units without a membrane and sealing pad, and flush with distilled water.

Note: Do not autoclave the ImmunoBlot unit or washing manifold.

Do not expose the ImmunoBlot unit to alcohol or other organic solvents.

Troubleshooting

problem	possible causes	recommendations
Leaking – antibody solution has moved to adjacent channels.	Dry areas of the membrane act as wicks for fluid from adjacent areas.	Always pre-wet the membrane before mounting in the unit. Fill every channel, covering the membrane with buffer.
	Membrane did not cover the full length of the channel.	Ensure that membrane is cut to proper size.
	Fluid in overloaded channels spilled out when unit was rocked causing contamination of adjacent channels.	Do not overfill channels.
	Re-used sealing pads did not seal properly.	Do not re-use sealing pads as they compress during use.
	Tray incubation.	Consider incubating your blot with secondary antibody in the ImmunoBlot rather than tray incubation. (See Appendix A: Note 6.)
	Membrane is stripped of blocking protein.	Please take care not to strip the blot of blocking protein while washing it prior to addition of primary antibodies. Please follow instructions from your Western blotting detection kit. A sample protocol is given in Appendix B.
	Unwashed channels contained antibodies that contaminated the experiment.	Clean the unit after every use. For cleaning tips, see the Clean the ImmunoBlot section, on page 7.
Low Sensitivity	When low affinity or low titer antibodies are used at high dilution (1:100,000 or greater), some decrease in signal intensity may be observed. Antibodies may become depleted in the small volume of the ImmunoBlot channel.	Increase the antibody concentration 2–5 fold. Increase the antibody volume solution per channel. To do this, insert standard 200 µl plastic pipette tips into both entry ports for each channel. These will serve as reservoirs for sample volumes larger than the volume of the channel itself, while incubating on the rocker. Inject the antibody sample using a tip that then remains in the entry port and becomes such a reservoir. Perform secondary antibody incubations in a tray. (See Appendix A, Note 6.)

problem	possible causes	recommendations
Bubbles (**Note:** Small bubbles generally move when the unit is rocked and do not usually affect results.)	Solutions were too cold. Dissolved gas emerges from the solution as the temperature rises.	Bring solutions to room temperature before loading samples.
	Channels contained residual drops of liquid before samples were loaded.	Tilt the unit and aspirate liquid from the lower end of each channel with a plastic pipette tip attached to a strong vacuum.
	Improper pipetting technique was used.	Seat the pipette tip firmly in the numbered hole and dispense the sample with a single smooth action.
	Sealing pads were moist before mounting.	Ensure that sealing pads are dry before mounting them on the ImmunoBlot.
	Poor quality or poorly maintained pipette introduced bubbles into the channels.	Try injecting samples with a different pipette or brand of tip.
	Bubbles formed over time.	Place a layer of plastic wrap between membrane and sealing pad.
Manifold is difficult to insert.	O-rings may need lubrication or replacement.	To lubricate: • Remove O-rings carefully with the tip of a flat weighing spatula. • Apply silicone grease lightly. • Reinstall.

Appendix A: Technical notes

Note 1: Blocking the membrane

Membranes should always be blocked *before* mounting in the ImmunoBlot. Please follow instructions for blocking provided in your Western blotting detection kit.

Generally, 5% Non-fat dry milk or 5% BSA are used as blocking agents.

Blocking with Tween-20 or other detergents alone is *not* recommended as it may cause slow lateral diffusion of proteins through the nitrocellulose membrane. We recommend performing the initial blocking step in a solution containing protein (such as 5% BSA) without detergent. Avoid extensive washes with solutions containing no protein prior to mounting the membrane in the ImmunoBlot.

In many cases, satisfactory blocking may be attained by incubation with PBS/10% newborn calf serum/0.1% Tween-20 for a minimum of one hour at room temperature. However, blocking agents differ in their efficacy depending upon circumstances. For blots of whole cell lysates, blocking with 50 to 100% serum can be very effective in reducing non-specific binding.

Note 2: Aligning the membrane

For Western blotting experiments it is important that lanes and protein bands on the membrane align with the channels of the ImmunoBlot unit. This can be accomplished in 2 steps:

Step 1.

Use the specially designed ImmunoBlot combs at the time of electrophoresis. This ensures that the lanes on the gel and, subsequently, the lanes and protein bands on the blot, are aligned with the channels on the ImmunoBlot.

Combs with well spacings that match the lane spacings of the ImmunoBlot units are listed below. Please note that the number of wells does not always match the number of lanes in a 1:1 ratio.

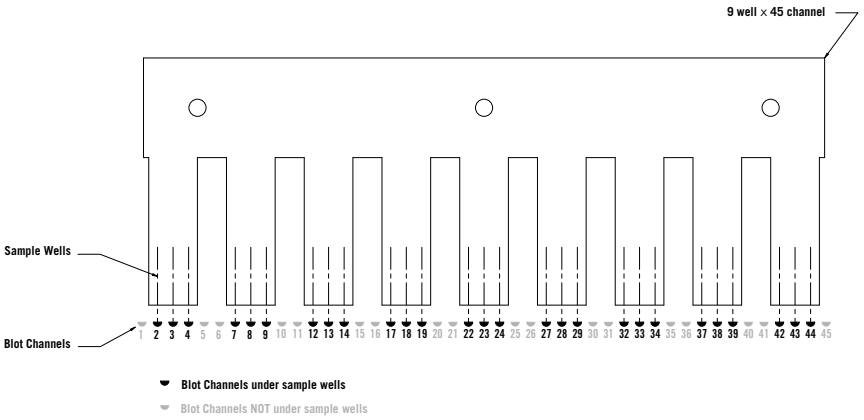
9 well × 45 channel
Sample Wells
Blot Channels
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45
Blot Channels under sample wells
Blot Channels NOT under sample wells

Fig A. The 9-well 1.0-mm comb (PR511-9-1.0) aligns with the 45 channels of the ImmunoBlot XL and can be used to probe 9 samples with up to three probes per sample.

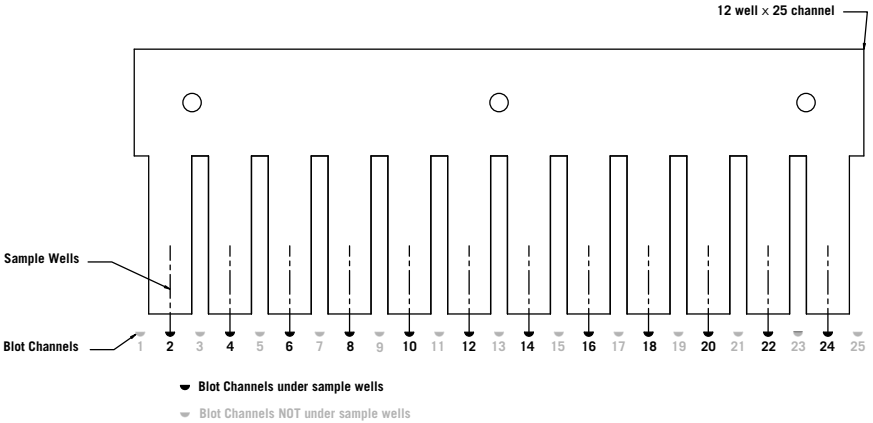
12 well x 25 channel
Sample Wells
Blot Channels
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Blot Channels under sample wells
Blot Channels NOT under sample wells

Fig B. The 12-well 1.0-mm comb (PR511-12-1.0) aligns with the 25 channels of the ImmunoBlot and can be used to probe 12 samples with one probe per sample.

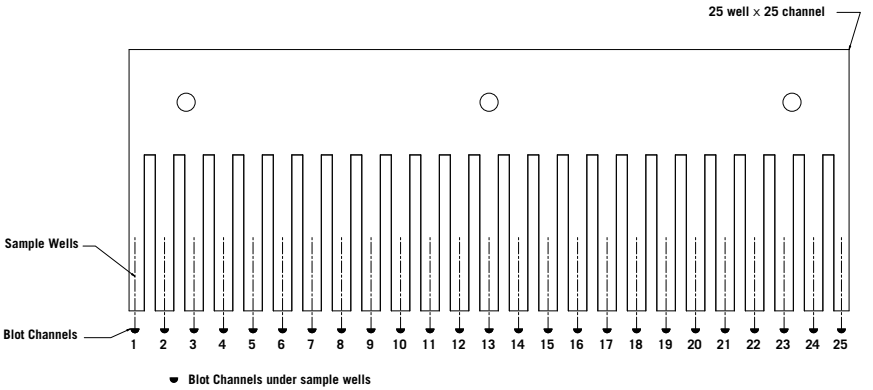
25 well x 25 channel
Sample Wells
Blot Channels
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Blot Channels under sample wells

Fig C. The 25-well 1.0-mm comb (PR511-25-1.0) also aligns with the 25 channels of the ImmunoBlot and can be used to probe 25 samples with one probe per sample.

Step 2.

After the gels are blotted, the bands on the membrane are not visible. Adding a non-reactive dye will make it easy to align the membrane. This can be done in one of two ways:

A. For larger protein loads (>250 ng) Ponceau S can be used to stain the proteins reversibly on the membrane following electrophoretic transfer.

   Rinse the membrane briefly in water and stain in 0.2% Ponceau S for 1 to 2 minutes, then destain in several changes of distilled water until red bands appear against a white background. Since the stain is lost during the subsequent blocking step, protein bands should be marked at this stage with pinholes or with a pointed pencil. The blot may also be photocopied.

   Ponceau S stained blots can be stored for months at 4 °C, kept moist between sheets of parafilm or buffer saturated filter paper. Blots can also be frozen in a plastic pouch.

   Ponceau S powder should be dissolved in distilled water to make a working solution. The solution can be reused for several weeks to months depending upon the number of membranes stained.

B. For lower protein loads (<250 ng), methyl green, Pyronin Y or Deep Purple may be used to permanently mark the top and bottom of the gel for subsequent alignment with the ImmunoBlot channels.

   When loading the gel, add approximately 5 µl of methyl green (0.1% solution in 50% glycerol) or Pyronin Y (0.05% solution in 50% glycerol) to the desired lanes. These dyes migrate just ahead of the bromophenol blue dye front in the gel, and will transfer permanently to the membrane. A second aliquot of the dye added to the gel near the end of the electrophoretic run will enter the resolving gel in 5 to 10 minutes and serve to mark the top of the gel. Please keep in mind that these dyes can interfere with fluorescent Western blotting detection methods and should therefore be removed prior to imaging. If left on the membrane they will result in nonspecific signals in fluorescent Western blotting detection.

Note 3. Eliminating bubbles

Small bubbles in the channels generally do not affect the end reaction, and should move back and forth over the surface of the membrane when the ImmunoBlot is rocked. To eliminate large bubbles, withdraw the solution from the channel and re-inject. For further information, refer to the Troubleshooting section.

Note 4. Optimal volumes

The optimal volume for the ImmunoBlot channels will vary slightly depending on the type of membrane used. The solution added should almost (95%) fill the entire channel. Reduce the volume if the solution spills out of the entry port when the unit is incubated on a rocking platform.

Note 5. Fitting the O-rings into the slots

If your washing manifold units do not fit into the slots easily, apply a small amount of silicone grease around the O-rings. Place the manifold unit loosely in the slot and press down on the side of the manifold towards you. Then press on the side away from you to seat the manifold firmly in the slot.

Note 6. Secondary antibody incubations

Please follow instructions provided with your Western blotting detection system. When secondary antibody is used in the ImmunoBlot at a high dilution (1:100,000 or greater), some decrease in signal intensity may be observed. This may be due to depletion of antibodies in the small volume of the channel. The problem can generally be corrected by 1) increasing the antibody concentration 2–5 times, 2) increasing the antibody volume, or 3) removing the membrane from the unit and performing the secondary antibody incubation in a tray.

Note: Exercise caution when performing tray incubations because low affinity monoclonal antibodies can dissociate from their respective antigens over time. Incubation in a tray permits such antibodies to diffuse through the incubation solution and rebind elsewhere on the blot. Such *streaking* is detectable as staining between sample lanes or in negative control lanes at the position of one or more strongly reactive bands.

Appendix B: Detection using ECL Western blotting detection systems

Hoefer recommends that you perform labeling and detection on blots following the instructions provided in your Western blotting labeling and detection kit. Below is a general protocol based on the GE Healthcare (formerly Amersham Biosciences) ECL Western Blotting Detection Kit.

There are several ECL Western blotting detection systems available.

The following general protocol is suggested:

❶

After electrophoresis and transfer of separated proteins to nitrocellulose or PVDF (low fluorescent Hybond LFP for highest sensitivity) membrane, block unspecific sites with a suitable blocking solution.

❷

Blocking is followed by two quick washes in PBS/0.1%Tween (PBS-T).

❸

Immerse and incubate the membrane with an antigen-specific primary antibody of optimized concentration.

❹

Remove the membrane with two quick washes of PBS-T followed by 2 longer washes (2 × 5 min with rocking).

❺

Incubate the membrane with an optimized concentration of the conjugated secondary antibody.

❻

Briefly rinse the membrane with two changes of wash buffer followed by 4 longer washes in PBS-T (4 × 5 min with rocking).

❼

Detection solution A and B are mixed and pipetted onto the membrane for a short incubation.

❽

Drain off excess detection reagent and wrap the membrane in Saran-Wrap before exposure to X-ray film.

Ordering information

product	quantity	code no.
ImmunoBlot, for use with standard sized gel membrane (*e.g.* SE600). 25 probe lanes spaced 5.3 mm apart. Includes top plate, bottom plate, 2 screws, washing manifold (2 pieces), 2 pieces of tubing, tubing connectors, and 5 sealing pads.	1	PR625
ImmunoBlot XL, for use with standard sized gel membrane (*e.g.* SE600). 45 probe lanes spaced 3.0 mm apart. Includes top plate, bottom plate, 2 screws, washing manifold (2 pieces), 2 pieces of tubing, tubing connectors, and 5 sealing pads.	1	PR645
Plastic Sealing Pads	10	PR630-31
Washing Manifold Kit	1	PR630-32
O-ring Seals	2	PR630-33
Manifold Luer Connectors	4	PR630-34
Clamp Screw	1	PR630-36
Top Plate for PR625	1	PR625T
Top Plate for PR645	1	PR645T
Bottom Plate	1	PR630-37
Comb, 9 well 1.0 mm	1	PR511-9-1.0
Comb, 12 well 1.0 mm	1	PR511-12-1.0
Comb, 25 well 1.0 mm	1	PR511-25-1.0

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